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A Novel Mutation of the Signal Peptide of the Preproparathyroid Hormone Gene Associated with Autosomal Recessive Familial Isolated Hypoparathyroidism*

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ABSTRACT

We report a novel mutation of the signal peptide of the prepro-PTH gene associated with autosomal recessive familial isolated hypoparathyroidism. The proposita presented with neonatal hypocalcemic seizures. Serum calcium was 1.5 mmol/L (normal, 2.0–2.5); phosphate was 3.6 mmol/L (normal, 0.9–1.5). She was born to consanguineous parents. A few years later, 2 younger sisters and her niece presented with neonatal hypocalcemic seizures. Their intact PTH levels were undetectable during severe hypocalcemia. Genomic DNA from the proposita was sequenced all exons of the prepro-PTH gene. A replacement of thymine with a cytosine was found in the first nucleotide of position 23 in the 25-amino acid signal peptide. This results in the replacement of the normal Ser (TCG) with a Pro (CCG). Genotyping of family members was carried out by identification of a new *MspI* site created by the mutation. Only affected family members were homozygous for the mutant allele, whereas the parents were heterozygous, supporting autosomal recessive inheritance. As this mutation is at the −3 position in the signal peptide of the prepro-PTH gene, we hypothesized that the prepro-PTH mutant might not be cleaved by signal peptidase at the normal position, and it might be degraded in rough endoplasmic reticulum. (*J Clin Endocrinol Metab* **84:** 3792–3796, 1999)

IDIOPATHIC hypoparathyroidism is a heterogeneous group of metabolic disorders characterized by hypocalcemia and hyperphosphatemia due to deficient secretion of PTH. Although most cases are sporadic, familial occurrence of idiopathic hypoparathyroidism has been reported as well. Familial hypoparathyroidism may also occur as part of a complex autoimmune disorder associated with multiple endocrine deficiencies or developmental defects. Familial hypoparathyroidism may occur as an isolated entity without associated abnormalities, and this form of the disorder is familial isolated hypoparathyroidism (FIH).

The human PTH gene contains 3 exons that located on the short arm of chromosome 11 (1). Exon 1 contains the untranslated region. Exon 2 encodes the signal peptide and part of the prohormone sequence. Exon 3 encodes the remainder of the prohormone sequence, the 84-amino acid PTH peptide, and the 3′-untranslated region. PTH is formed as a larger prepro-PTH. This precursor undergoes 2 successive proteolytic cleavages to yield PTH. The signal peptide is cleaved first during cotranslational translocation, releasing the pro-PTH into the lumen of the rough endoplasmic reticulum (RER). Pro-PTH is processed later in the Golgi apparatus to produce the mature PTH (2).

Mutations in the PTH gene have been reported in only 2 families with FIH. The first family had mutation in the hydrophobic core of the signal peptide, producing the autosomal dominant form of FIH (3). The second family had a mutation in the exon 2-intron 2 junction that skipped the next exon and produced the autosomal recessive form of FIH (4). In this paper we described a new autosomal recessive FIH associated with a point mutation at the −3 position (counting from the cleavage site between positions −1 and +1) in the signal peptide of the prepro-PTH gene that leads to an amino acid substitution, Ser to Pro. We have established that the prepro-PTH gene allele bearing the observed mutation is linked to the FIH phenotype.

Subjects and Methods

Patients

The proband (subject IV-10) was born in 1975 and presented at 7 days of age with seizures (Fig. 1A). On the presumption of epilepsy, she was treated with anticonvulsive agents for several months, but seizures continued. Subsequent investigation at the Queen Sirikit National Institute of Child Health revealed hypocalcemia at 1.5 mmol/L (normal range, 2.0–2.5), with hyperphosphatemia at 3.6 mmol/L (normal range, 0.9–1.5), and the diagnosis of isolated hypoparathyroidism was made. Serum calcium levels were maintained with vitamin D and calcium therapy. She died by drowning at 10 yr of age. Subsequently, it was learned that the parents (subjects III-3 and III-4) were consanguineous.

Her younger sister (subject IV-11) was born in 1978 and presented in infancy with hypocalcemic seizures. Serum calcium was 1.5 mmol/L, and phosphate was 2.9 mmol/L, suggesting the diagnosis of FIH. She was treated with large doses of vitamin D (vitamin D_2, 120,000 U/day) and calcium (calcium lactate, 7 g/day). At the last evaluation at age 20 yr, serum calcium was 1.5 mmol/L, phosphate was 2.1 mmol/L, and serum intact PTH (iPTH) measured by immunoradiometric assay (ELSA-PTH, CIS Biointernational, Gif-Sur-Yvette cedex France) was

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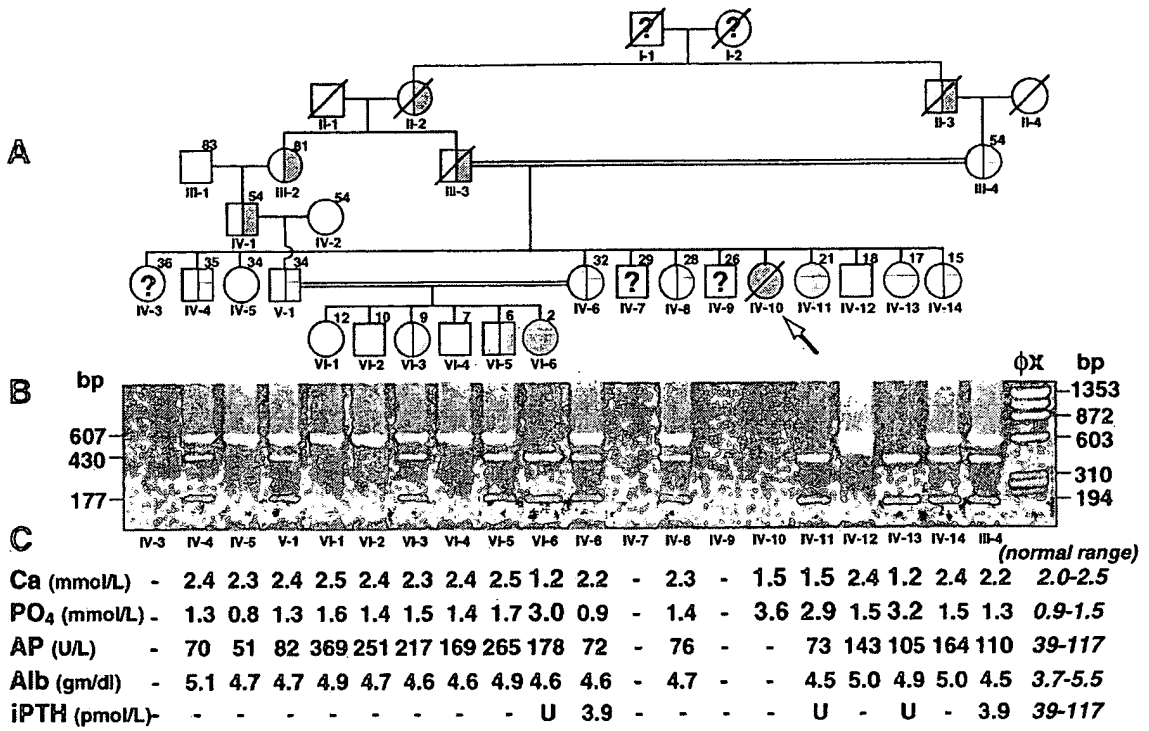

FIG. 1. Pedigree of the family, genotyping for the prepro-PTH gene mutation, and results of serum calcium, phosphate, alkaline phosphatase, and intact PTH levels. A, The family pedigree and transmission of the phenotype are compatible with the autosomal recessive mode of inheritance. *Black symbols* indicate affected subjects who are homozygous for the mutation. *Half-black symbols* indicate individuals heterozygous for the mutation. *Shaded symbols* and *half-shaded symbols* indicate individuals suspected of being homozygous and heterozygous for the mutation, respectively; they were not tested for genotyping. The proband is indicated by an *arrow*. Generations are in *Roman numerals*, and individuals are in *Arabic numbers*. Ages are on the *right* of the symbols. B, A 607-bp fragment of exons 2–3 of the PTH gene was amplified from genomic DNA and digested with *MspI*. Digestion into 430- and 177-bp DNA fragments denoted the presence of the mutation. All affected family members who have both mutant alleles show complete digestion of the 607-bp band. C, Results of serum calcium, phosphate, alkaline phosphatase, albumin, and intact PTH levels are aligned with individuals' symbols. All affected family members had severe hypocalcemia, hyperphosphatemia, and undetectable intact PTH levels. Values outside the normal range for age are in *bold numbers*.

undetectable (normal range, 0.8–7.6 pmol/L; detection limit, 0.3 pmol/L). Serum alkaline phosphatase was 73 U/L (normal range, 39–117); serum creatinine was 62 μmol/L (normal range, 44–177). Her height was 146 cm (3–10th percentile). Physical examination was normal, except for positive Chvostek's sign.

In 1982, her younger sister (subject IV-13) was born and presented with neonatal hypocalcemic seizures. Serum calcium was 1.1 mmol/L; phosphate was 3.3 mmol/L. She was also treated with large doses of vitamin D and calcium. At age 16 yr, serum calcium was 1.2 mmol/L, phosphate was 2.8 mmol/L, alkaline phosphatase was 105 U/L, and serum iPTH was undetectable. Her height was 148 cm (10–25th percentile). Physical examination was normal, except for positive Chvostek's sign. Three of 12 siblings of the consanguineous couple, III-3 and III-4, had FIH (Fig. 1A). Six of them (subjects IV-4, IV-5, IV-6, IV-8, IV-12, and IV-14) were examined and found to be unaffected, as was their mother (subject III-4). Their serum calcium and phosphate levels were normal, and the iPTH level of their mother (subject III-4) was normal (3.93 pmol/L). We were unable to examine or test 3 of the siblings (subjects IV-3, IV-7, and IV-9) and their father (subject III-3), but they were reported to be healthy and had no history of seizures.

In the next generation, the niece of the propositus (subject VI-6), also born of consanguineous parents (subjects V-I and IV-6), presented with neonatal hypocalcemic seizures. Serum calcium was 1.2 mmol/L, phosphate was 2.8 mmol/L, alkaline phosphatase was 178 (normal range, 110–360), and serum iPTH was undetectable. In this generation (Fig. 1), her five siblings (subjects VI-1, VI-2, VI-3, VI-4, and VI-5) as well as their parents (subjects V-1 and IV-6) were normocalcemia and had no history of seizures. The serum iPTH level of her mother was normal (3.88 pmol/L).

The inheritance of FIH in this family is autosomal recessive. No family member had evidence of mucocutaneous candidiasis, autoimmune endocrine disease, or somatic features consistent with a developmental or embryological disorder. This family was referred to Rajavithi Hospital for further investigations.

Preparation of genomic DNA and DNA sequencing

Genomic DNA of affected subject IV-11 was isolated from peripheral blood leukocytes using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI). The genomic DNA was used as a template for PCR amplification of exon 1 of the prepro-PTH gene and a region extending from exon 2 through exon 3 covering the coding regions and splice junctions. Primer sequences for PCR amplification of exon 1 were 5′-ctctcttggtaagcagaaga-3′ (sense) and 5′-ccttgaagaaacaacatggt-3′ (antisense). Primer sequences for exons 2–3 were 5′-gcttctcgtgaaaaccaacc-3′ (sense) and 5′-ccctacactgtctagagcag-3′ (antisense). The conditions for amplification by PCR were 100 μL containing 0.2 μg genomic DNA, 100 pmol of each primer, 200 μmol/L of each deoxy-NTP, 2.5 mmol/L $MgCl_2$, 5 mmol/L Tris-HCl (pH 8.0), 10 mmol/L NaCl, 10 μmol/L ethylenediamine tetraacetate, 0.5 mmol/L dithiothreitol, 5% glycerol, 0.1% Triton X-100, and 0.8 U *Taq* DNA polymerase (Promega Corp.). Initial denaturation was performed at 94 C for 5 min, followed by 35 cycles of 94 C for 1 min, 58 C for 1 min, and 72 C for 1 min and a final extension at 72 C for 15 min. The amplified DNA fragment was sequenced using a 373 DNA Sequencer (PE Applied Biosystems, Perkin Elmer Corp., Foster City, CA).

Confirmation of the mutation

To confirm the presence of the mutant nucleotide in genomic DNA and to identify family members who harbored the mutation, we amplified exons 2 and 3 of the prepro-PTH gene of the subjects' genomic DNA as described above. As the mutation in position 23 of the signal peptide, a replacement of thymine by cytosine, creates a new recognition site for *Msp*I (CCGG), this endonuclease was used to digest the amplified 607-bp fragment. The presence of the mutant cytosine generates two fragments of 430 and 177 bp detected by electrophoresis on a 2% agarose gel (Fig. 1B). Partial or complete cleavage of the DNA fragment indicated that the mutant nucleotide was present in one or both alleles, respectively. The experiments were performed at least twice, and the results were reproducible. Each experiment had affected patient's DNA that was homozygous for the mutation to ensure that all samples were completely digested by restriction enzyme.

A total of 16 individuals were tested for genotyping (Fig. 1A). Subject IV-10 was the only affected patient who was given a biochemical test, but she died before genotyping. In generation IV, there were 12 siblings: 8 siblings received physical examination, biochemical test, and genotyping; 1 sibling (subject IV-10) received physical examination and biochemical test, except genotyping; and 3 siblings received neither physical examination nor any test. In generation VI, all members were tested for genotyping.

To predict the signal sequence cleavage site probability

We applied the method of von Heijne (5) to predict the locations of signal peptide cleavage sites and alternative cleavage sites by comparing wild-type prepro-PTH sequence with the sequence of the prepro-PTH mutant. On a weight-matrix approach, this method can identify the correct cleavage site about 75–80% of the time when applied to new sequences. This −3,−1 rule combined with the expected distribution of other amino acids within the cleavage domain (−13 to +2) have been used to construct a weight matrix to calculate the probability of cleavage at a specific site.

Results

The subjects affected by FIH were 3 of 12 siblings in generation IV and 1 of 6 siblings in generation VI, all born to consanguineous parents. The inheritance pattern is autosomal recessive (Fig. 1A). Affected family subjects had severe hypocalcemia and hyperphosphatemia, whereas the unaffected members were healthy, and their serum calcium and phosphate levels were normal. The serum iPTH levels of all symptomatic patients were undetectable despite severe hypocalcemia. The data explain why the severe hypocalcemia was very difficult to correct with calcium and vitamin D supplementation.

DNA sequencing revealed normal DNA sequence of exon 1 of the prepro-PTH gene. We found a mutation in exon 2 located at the first nucleotide of position 23 in the 25-amino acid signal peptide (Fig. 2B). A thymine (TCG) was substituted by a cytosine (CCG), resulting in the replacement of the normal Ser by Pro (Fig. 2B). This mutation in the prepro-PTH gene was confirmed by digestion with *Msp*I, a new endonuclease recognition site created by the mutation. The result showed that all affected members were homozygous for the mutant allele, and their parents were heterozygotes in agreement with the autosomal recessive mode of inheritance. In

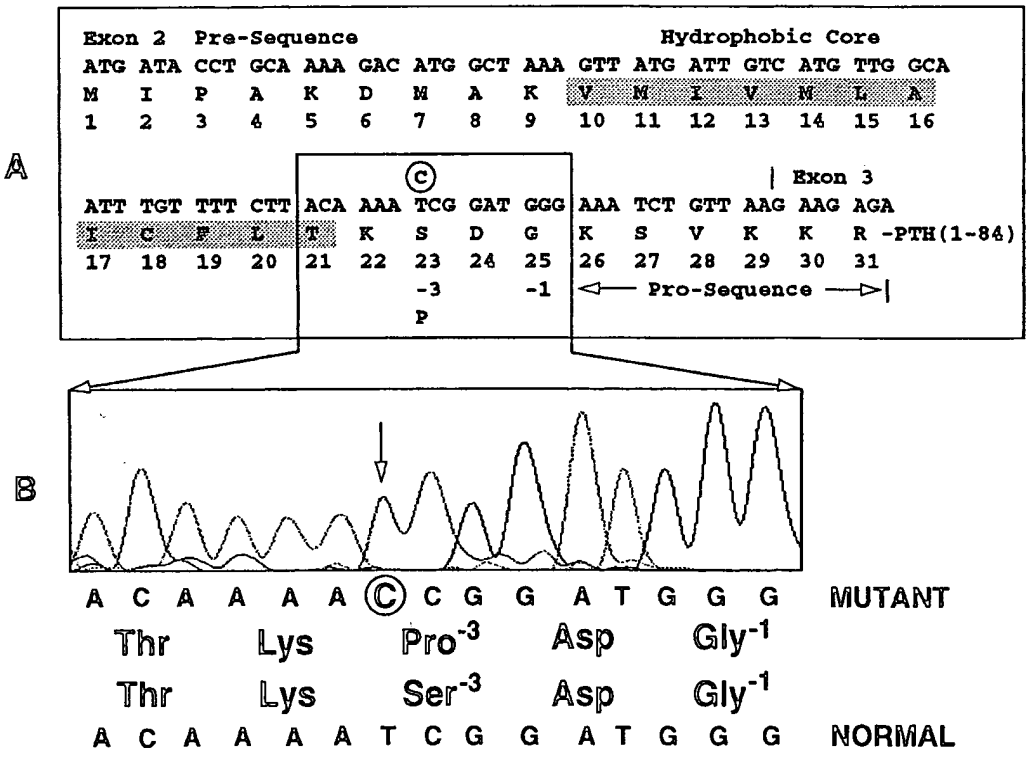

FIG. 2. Nucleotide and amino acid sequences of the signal pre and pro regions of prepro-PTH. A, Amino acids 1–25 comprise the signal peptide; residues 26–31 constitute the pro sequence, and the remaining 84 amino acids are mature PTH (not shown). Amino acids 10–21, comprising the hydrophobic core of the signal peptide, are in a *shaded box*. The described patient's mutation at position 23 is indicated by an *arrow*. B, Segment of DNA sequence showing the mutation in the prepro-PTH gene. The mutation was found at the first nucleotide of position 23 in the 25-amino acid signal peptide. A thymine was substituted by a cytosine, resulting in the replacement of the normal Ser (TCG) with a Pro (CCG).

generation IV, we found the mutation in only 6 of the 8 subjects tested: 2 were affected homozygotes, and 4 were heterozygotes and clinically normal. Two normal subjects had no mutation in either allele. In generation VI, there were 6 children: 1 affected homozygote for the mutant allele, 2 heterozygotes for the mutation, and 3 with both normal alleles. The latter as well as the heterozygotes had normal serum calcium and phosphate levels.

The probability matrix of von Heijne was used to assign cleavage site probabilities to the residues in the expected cleavage domains of wild-type and mutant PTH proteins. The more positive score has the highest probability for being the cleavage site. The result showed that the mutant prepro-PTH protein had a very low positive score at a normal cleavage position (Fig. 3), which suggested that signal peptidase is unlikely to cleave the mutant prepro-PTH protein.

Discussion

Signal sequences are present within the precursors of most secreted proteins and are required to direct these proteins into the cell's secretory pathway. Signal recognition particles (SRPs) bind signal sequences and then bind to the SRP receptor (docking protein) (6) in the microsomal membrane, thus delivering the precursor protein to the outer border of the RER. The precursor protein is then inserted into the membrane of the RER and translocated; the signal sequence is cleaved by a membrane-bound enzyme, signal peptidase (7). The peptide is then transported through a series of membrane-bound compartments that include the various cisternae of the Golgi complex, the *trans*-Golgi network, and secretory granules (8).

Signal peptides typically are made up of three domains, consisting of a positively charged NH_2-terminal region, a central hydrophobic region, and a polar COOH-terminal region (9, 10). The NH_2-terminal region may have something to do with the docking protein and is important for translocation. The central hydrophobic region is believed to be the target for the SRP (11). The COOH-terminal region influences the efficiency and fidelity of signal peptidase cleavage (9, 10).

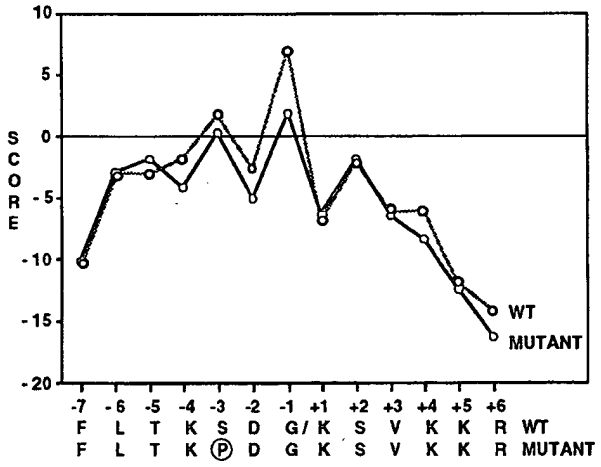

FIG. 3. Probability that indicated residues represent signal cleavage sites. The probability matrix of von Heijne was used to assign cleavage site probabilities to the residues in the expected cleavage domains of wild-type and mutant PTH proteins. Amino acid residues are indicated by *single letter code*, starting with residue 19 (position −7) of signal peptide PTH protein. Processing probability values are assigned to a given residue, with signal peptidase cleavage occurring between that residue and the one preceding it. A *slash line* indicates the border of the signal peptide and mature PTH.

Prepro-PTH has a 25-residue signal sequence, followed by a 6-residue propeptide sequence and an 84-residue of mature hormone (Fig. 2A). Structural features of signal peptide are critical for the translocation of secretory proteins and their cleavage by signal peptidase (12). The COOH-terminal region, which could introduce flexibility into the molecule, may allow signal peptidases to adopt a loop or hairpin structure near the signal cleavage site in the membrane. The hairpin configuration has been proposed as the structure appropriate for insertion of the precursor into the membrane (13, 14) and for presenting an appropriate substrate to the signal peptidase (15). Alteration near the cleavage site can disrupt signal peptidase cleavage (15, 16). The human propeptide (Lys-Ser-Val-Lys-Lys-Arg) is cleaved from pro-PTH just before secretion, presumably in the *trans*-Golgi tubular network. Processing occurs after the dibasic residues Lys-Arg. Pro-PTH is not secreted from cells, and neither the pro-specific fragment nor any of the its possible degradation products accumulate in the cell (2). Thus, any role for the propeptide must be an intracellular one.

We described herein a point mutation at the first nucleotide of position 23 in the signal peptide-encoding region of a prepro-PTH gene in FIH. A thymine was substituted by a cytosine, resulting in replacement of the normal Ser (TCG) by Pro (CCG). Consanguinity and family size establish the autosomal recessive mode of inheritance. This mutation is conceivably the cause of the hypoparathyroidism in affected members of this family, because genotyping shows that inheritance of PTH deficiency is tightly linked to the mutant allele. Furthermore, the mutation is located in the crucial position for signal peptidase cleavage site. The prepro-PTH 23 (Ser→Pro) mutation was found in both alleles of the affected patients, and their parents were heterozygous for the mutation.

The mutation corresponds to the −3 position of the prepro-PTH protein cleavage site. According to the −3,−1 rule of the signal peptidase recognition site (10, 12), the region around the cleavage site shows strong preferences for specific amino acids in particular positions. Acceptable cleavage domains conform to the following rules: the residue at position −1 from the cleavage site must be small (Ala, Ser, Gly, Cys, Thr, or Gln); the residue at position −3 must not be aromatic (Phe, His, Tyr, and Trp), charged (Asp, Glu, Lys, and Arg), or large polar residues (Asn and Gln); and there must be no Pro residue in the region between −3 and +1 position (10, 12). The −3,−1 positions of signal sequence are crucial for signal peptidase to cleave prepro-PTH to pro-PTH protein in the RER. The change at position −3 of Ser for Pro has never been encountered, and Pro is a strong helix-breaking residue. We, therefore, hypothesize that the prepro-PTH 23 (Ser→Pro) mutation might exert its dramatic effect on signal function by interfering with signal peptidase cleavage. If the signal sequence is not cleaved, the prepro-PTH mutant may anchor in the microsomal membrane, and eventually it might be degraded in the RER (13, 17, 18), it may pass com-

pletely through the membrane (19), or it could have a new signal peptidase cleavage site downstream and make new pro-PTH protein. The process might impair the release of PTH molecules from the parathyroid gland to circulation because of the absence of PTH in the affected patients during hypocalcemia. Unfortunately, we were unsuccessful in expressing the prepro-PTH gene, either wild type or mutant, to support our hypothesis.

The mutation in signal peptide that closely relates to our mutation is coagulation factor $X_{Santo\ Domingo}$ (FXsd) (21). FXsd is a mutant form of human factor X in which a point mutation results in the substitution of Arg for Gly at the critical −3 position of the signal peptide (21). The patient bearing the mutation exhibits a severe bleeding diathesis associated with less than 1% FX enzymatic activity and less than 5% circulating FX protein. The mutation does not interfere with targeting and translocation to the RER, but cleavage by signal peptidase is dramatically impaired (22). It should be noted that this mutation does not induce a shift in the signal peptidase cleavage site, an effect that has been observed in other cases. Signal peptidase appears to have some degree of flexibility in its selection of cleavage site if a suitable alternative site is present. In the case of prepro-FXsd, it appears that a suitable alternative cleavage site is not available, so the result of the mutation is to block cleavage completely. Similarly, in the case of the prepro-PTH-23 (Ser→Pro) mutation, we hypothesized that this mutation can block signal peptide cleavage completely. To support this hypothesis, we used von Heijne's probabilistic method (5) to define the alternate cleavage site of the mutant precursor peptide. The method allows comparison of the mutant precursor to sequences of other characterized precursor proteins to predict appropriate cleavage sites. The probability of alternative cleavage site is extremely low in mutant peptide. It appears that a suitable alternative cleavage site is not available, so the result of the mutation is to block cleavage completely.

In the family reported herein, the inheritance is autosomal recessive, which contrasts with previous reports of mutations in the signal sequence of human secreted proteins that appear to have dominant inheritance (3, 20, 22). The affected patients containing the mutation in both alleles of prepro-PTH gene had no detectable PTH in the circulation that favors lack of PTH secretion from parathyroid glands. Their parents and heterozygous siblings, who had one mutant and one normal prepro-PTH allele, were clinically normal and normocalcemic and had normal levels of serum iPTH. The apparent ability of only one normal prepro-PTH allele to maintain PTH secretion is sufficient amount to prevent hypocalcemia and maintain calcium homeostasis, which is compatible with other case of FIH reported by Parkinson *et al.* (4). Their mutation involved a donor splice site mutation at the exon 2-intron 2 boundary that caused exon skipping, and the inheritance is autosomal recessive (4). In contrast, Arnold *et al.* (3) reported a dominantly inherited FIH associated with the substitution of Arg for Cys within the hydrophobic core of prepro-PTH. The mutation causes a disruption of the core that leads to impair interaction of the nascent protein with SRP, the translocation machinery, and signal peptidase cleavage (23). Hypoparathyroidism in the presence of one normal PTH allele would therefore suggest that the mutant gene product exerts a dominant negative effect *in vivo*. Although our mutation is in signal peptide of prepro-PTH gene, the mutation might interfere only with signal peptidase cleavage, and the mutant gene product might not interfere with normal PTH production from the normal prepro-PTH allele.

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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

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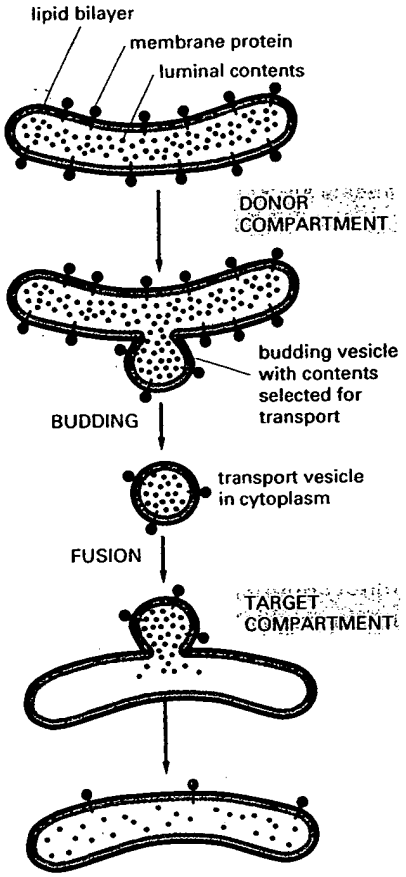

Figure 12–6 The "sidedness" of membranes is preserved during vesicular transport. Note that the original orientation of both proteins and lipids in the donor-compartment membrane is preserved in the target-compartment membrane and that soluble molecules are transferred from lumen to lumen.

through the membrane. The initial transport of selected proteins from the cytosol into the ER lumen or into mitochondria, for example, occurs in this way. (3) In **vesicular transport,** *transport vesicles* ferry proteins from one compartment to another. The vesicles become loaded with a cargo of molecules derived from the lumen of one compartment as they pinch off from its membrane; they discharge their cargo into a second compartment by fusing with its membrane. The transfer of soluble proteins from the ER to the Golgi apparatus, for example, occurs in this way. Because the transported proteins do not cross a membrane, they move only between compartments that are topologically equivalent (Figure 12–6). We discuss vesicular transport in more detail in Chapter 13. The three ways in which proteins are transported between different compartments are summarized in Figure 12–7.

Each of the three modes of protein transfer is usually selectively guided by sorting signals in the transported protein that are recognized by complementary receptor proteins in the target organelle. If a large protein is to be imported into the nucleus, for example, it must possess a sorting signal that is recognized by receptor proteins associated with the nuclear pore complex. If a protein is to be transferred directly across a membrane, it must possess a sorting signal that is recognized by the translocator in the membrane to be crossed. Likewise, if a protein is to be incorporated into certain types of transport vesicles or to be retained in certain organelles, its sorting signal must be recognized by a complementary receptor in the appropriate membrane.

Signal Peptides and Signal Patches Direct Proteins to the Correct Cellular Address [4]

There are at least two types of sorting signals on proteins. One type resides in a continuous stretch of amino acid sequence, typically 15 to 60 residues long. This **signal peptide** is often (but not always) removed from the finished protein by a specialized **signal peptidase** once the sorting process has been completed. The other type consists of a specific three-dimensional arrangement of atoms on the

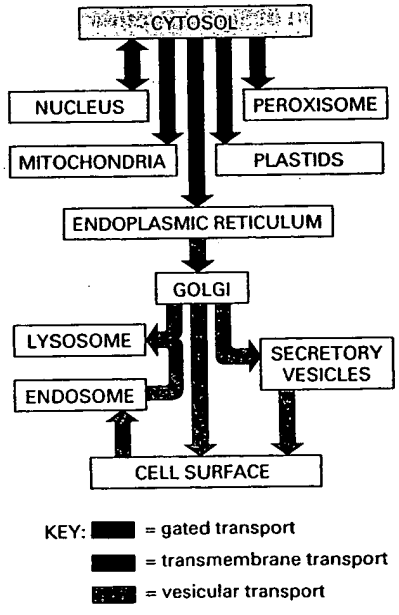

Figure 12–7 A simplified "road map" of protein traffic. Proteins can move from one compartment to another by gated transport (*red*), transmembrane transport (*blue*), or vesicular transport (*green*). The signals that direct a given protein's movement through the system, and thereby determine its eventual location in the cell, are contained in its amino acid sequence. The journey begins with the synthesis of a protein on a ribosome and terminates when the final destination is reached. At each intermediate station (*boxes*) a decision is made as to whether the protein is to be retained or transported further. In principle, a signal could be required either for retention in or for exit from each of the compartments shown, with the alternative fate being the *default pathway* (one that requires no signal). The vesicular transport of proteins from the ER through the Golgi apparatus to the cell surface, for example, appears not to require any specific sorting signals; specific sorting signals therefore are required to retain in the ER and the Golgi apparatus those specialized proteins that are resident there.

We shall use this figure repeatedly as a guide throughout this chapter and the next, highlighting the particular pathway being discussed.

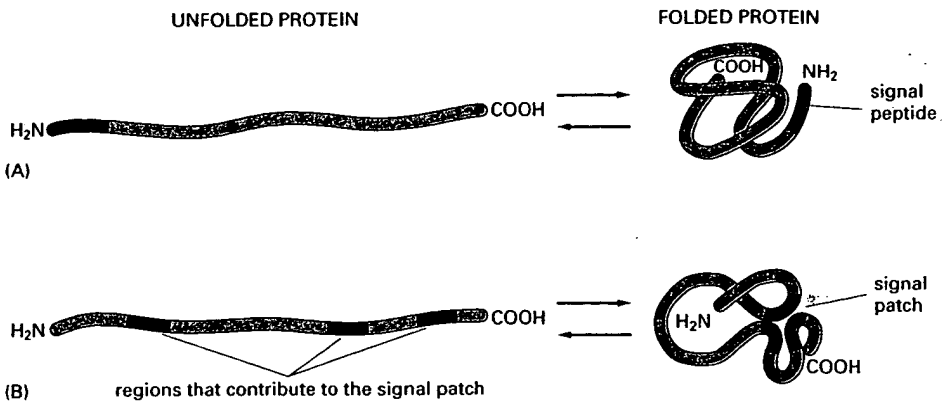

Figure 12–8 Two ways that a sorting signal can be built into a protein. The signal resides in a single discrete stretch of amino acid sequence, called a *signal peptide*, that is exposed in the folded protein. Signal peptides often occur at the end of the polypeptide chain (as shown), but they can also be located elsewhere. (B) A *signal patch* can be formed by the juxtaposition of amino acids from regions that are physically separated before the protein folds (as shown); alternatively, separate patches on the surface of the folded protein that are spaced a fixed distance apart could form the signal. In either case the transport signal depends on the three-dimensional conformation of the protein, which makes it difficult to locate the signal precisely.

protein's surface that forms when the protein folds up. The amino acid residues that comprise this **signal patch** can be distant from one another in the linear amino acid sequence, and they generally remain in the finished protein (Figure 12–8). Signal peptides are used to direct proteins from the cytosol into the ER, mitochondria, chloroplasts, peroxisomes, and nucleus, and they are also used to retain soluble proteins in the ER. Signal patches identify certain enzymes that are to be marked with specific sugar residues that then direct them from the Golgi apparatus into lysosomes; signal patches are also used in other sorting steps that have been less well characterized.

Different types of signal peptides are used to specify different destinations in the cell. Proteins destined for initial transfer to the ER usually have a signal peptide at their amino terminus, which characteristically includes a sequence composed of about 5 to 10 hydrophobic amino acids. Most of these proteins will in turn pass from the ER to the Golgi apparatus, but those with a specific sequence of four amino acids at their carboxyl terminus are retained as permanent ER residents. Proteins destined for mitochondria have signal peptides of yet another type, in which positively charged amino acids alternate with hydrophobic ones. Proteins destined for peroxisomes usually have a specific signal sequence of three amino acids at their carboxyl terminus. Many proteins destined for the nucleus carry a signal peptide formed from a cluster of positively charged amino

Table 12–3 Some Typical Signal Peptides

Function of Signal Peptide	Example of Signal Peptide
Import into ER	$^{+}H_3N$-Met-Met-Ser-Phe-Val-Ser-Leu-Leu-Leu-Val-Gly-Ile-Leu-Phe-Trp-Ala-Thr-Glu-Ala-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Gln-
Retain in lumen of ER	-Lys-Asp-Glu-Leu-COO^{-}
Import into mitochondria	$^{+}H_3N$-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-
Import into nucleus	-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-
Import into peroxisomes	-Ser-Lys-Leu-
Attach to membranes via the covalent linkage of a myristic acid to the amino terminus	$^{+}H_3N$-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-

Positively charged amino acids are shown in *red* and negatively charged amino acids in *green*. An extended block of hydrophobic amino acids is enclosed in a *yellow* box. H_3N^{+} indicates the amino terminus of a protein; COO^{-} indicates the carboxyl terminus.

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Signal peptide cleavage of a type I membrane protein, HCMV US11, is dependent on its membrane anchor

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The human cytomegalovirus (HCMV) US11 polypeptide is a type I membrane glycoprotein that targets major histocompatibility complex (MHC) class I molecules for destruction in a proteasome-dependent manner. Although the US11 signal sequence appears to be a classical N-terminal signal peptide in terms of its sequence and cleavage site, a fraction of newly synthesized US11 molecules retain the signal peptide after the N-linked glycan has been attached and translation of the US11 polypeptide has been completed. Delayed cleavage of the US11 signal peptide is determined by the first four residues, the so-called n-region of the signal peptide. Its replacement with the four N-terminal residues of the H-2K^b signal sequence eliminates delayed cleavage. Surprisingly, a second region that affects the rate and extent of signal peptide cleavage is the transmembrane region close to the C-terminus of US11. Deletion of the transmembrane region of US11 (US11-180) significantly delays processing, a delay overcome by replacement with the H-2K^b signal sequence. Thus, elements at a considerable distance from the signal sequence affect its cleavage.

Keywords: ER subdomains/HCMV US11/post-translational ER processing/signal sequence cleavage/transmembrane anchor

Introduction

Membrane proteins and proteins destined for secretion are targeted to the appropriate intracellular membrane by their signal peptides (Martoglio and Dobberstein, 1998). In eukaryotes, signal peptides are 15–50 amino acids long and are usually located at the N-terminus (von Heijne, 1983). A typical signal peptide is comprised of three distinct regions: a polar N-terminal end (n-region) that may have a net positive charge, a central hydrophobic core (h-region) that consists of 6–15 hydrophobic amino acids, and a polar C-terminal (c-region) end that contains prolines and glycines (von Heijne, 1985). A signal peptide containing the consensus sequence and proper cleavage site ensures that proteins are inserted into the endoplasmic reticulum (ER) membrane and are processed properly. Mutations within the sequence immediately downstream of the signal peptide affect protein processing, and can result in both inefficient and inaccurate cleavage (Russel and Model, 1981; Folz and Gordon, 1986; Andrews *et al.*, 1988; Wiren *et al.*, 1988). For example, replacement of glutamic acid for leucine at the +2 position of the phage coat protein cleavage site causes inefficient removal of its signal peptide (Russel and Model, 1981). When the propeptides of human pre-pro-apolipoprotein A-II and pre-pro-parathyroid hormone are deleted, five and six residues, respectively, the generation of an improper N-terminus and a failure to direct the nascent chain to the ER properly are observed (Folz and Gordon, 1986; Wiren *et al.*, 1988). Elements of the nascent chain at greater distances from the signal peptide are not known to affect signal peptide processing.

Shortly after its translation, the signal peptide interacts with signal recognition particle (SRP) and causes translational arrest (Walter and Blobel, 1981; Walter and Johnson, 1994). SRP is a ribonucleoprotein comprised of a 7S RNA associated with six different polypeptides (Walter and Blobel, 1980, 1982). The 54 kDa subunit of SRP interacts with the signal peptide through a hydrophobic region that promiscuously accommodates signal peptides of different lengths and sequences (Keenan *et al.*, 1998). The SRP–nascent polypeptide chain–ribosome complex is targeted to the ER membrane where SRP binds to the SRP receptor and the ribosome weakly interacts with the translocon (mainly comprised of the Sec61p complex) (Gorlich *et al.*, 1992; Kalies *et al.*, 1994; for reviews see Rapoport *et al.*, 1996; Hegde and Lingappa, 1999; Johnson and van Waes, 1999). The signal peptide is then transferred from the SRP into the channel of the translocon, where it directly associates with the Sec61α subunit of the Sec61 complex to promote tight interaction of the ribosome–nascent chain complex with the translocon (Jungnickel and Rapoport, 1995; Mothes *et al.*, 1998; Plath *et al.*, 1998). The signal peptide can also associate with the lipid bilayer and the TRAM protein (Martoglio *et al.*, 1995; Voigt *et al.*, 1996; Mothes *et al.*, 1997), which assists in protein transport through the translocon. The interaction of the signal peptide with the Sec61 complex may also induce the removal of a 'gating factor', possibly BiP, from the lumenal side of the translocon, to allow access of the nascent polypeptide to the ER lumen (Crowley *et al.*, 1994; Hamman *et al.*, 1998). Chain elongation is re-initiated, followed by signal peptide translocation through the Sec61 channel. The hydrophobic nature of the signal peptide allows its insertion into the ER membrane, followed by signal peptidase cleavage upon lumenal exposure of the cleavage site (Blobel and Dobberstein, 1975). This cleavage site is characterized by small uncharged residues at positions –1 and –3 (von Heijne, 1990). After signal peptide cleavage,

chain elongation of the nascent chain continues, while the signal peptide itself can be cleaved further by aminopeptidases or signal peptide peptidase (Lyko *et al.*, 1995; Martoglio *et al.*, 1997).

Signal peptidase is an endopeptidase that resembles other serine proteases (Dalbey and von Heijne, 1992) and performs a similar cleavage reaction for prokaryotic and eukaryotic signal peptidases. The crystal structure of the periplasmic domain of *Escherichia coli* leader peptidase (Paetzel *et al.*, 1998) reveals important mechanistic aspects of signal peptide cleavage: the catalytic site proposed to be close to the lipid bilayer is surrounded by a hydrophobic region, explaining the requirement for small uncharged, aliphatic residues at the −1 and −3 positions of the cleavage site (Paetzel *et al.*, 1998; von Heijne, 1998). The mammalian signal peptidase complex (SPC) is comprised of at least five subunits with molecular masses of 25, 23/22, 21, 18 and 12 kDa (Evans *et al.*, 1986). The non-catalytic subunits of the eukaryotic SPC may function as regulatory subunits for signal peptide recognition and are located in close proximity to the translocon (Meyer and Hartmann, 1997). The Sec61p complex interacts with the 25 kDa subunit of the SPC (SPC25), which suggests a tight interaction between the SPC and the Sec61 complex (Kalies *et al.*, 1998). This interaction may serve to recruit the SPC to the translocation site and thereby enhance the overall translocation efficiency of the nascent polypeptide.

The human cytomegalovirus (HCMV) gene products US11 and US2 target the major histocompatibility complex (MHC) class I molecules for destruction by the proteasome (Wiertz *et al.*, 1996a,b; Tortorella *et al.*, 1998). These viral proteins associate with the class I molecules in the ER and induce the dislocation of the class I heavy chains from the ER, probably via the Sec61p complex, for degradation in the cytosol (Wiertz *et al.*, 1996b). In all likelihood, a similar set of reactions is utilized for the removal and degradation of misfolded and abnormal ER proteins more generally (Bonifacino and Weissman, 1998). The HCMV US11 gene product is an ER-resident type I membrane glycoprotein (Figure 1), the single N-linked glycan attachment site of which is glycosylated quantitatively. The hydrophobic stretch at the N-terminus of US11 is characteristic of a signal peptide, while the hydrophobic stretch at the C-terminal end corresponds to a transmembrane/stop transfer sequence.

Here we report a highly unusual cleavage pattern for the US11 signal peptide. At least a fraction of the US11 signal peptide appears to be cleaved post-translationally. This trait is determined by the US11 signal peptide n-region. What cleavage occurs is also strongly influenced by the US11 transmembrane domain. Delayed cleavage of the US11 signal peptide may reflect the local ER environment in which dislocation takes place.

Results

The HCMV US11 signal peptide is cleaved post-translationally

HCMV US11 is a 215 residue ER-resident protein that targets MHC class I heavy chains for destruction by the proteasome. The detailed mechanism by which the viral

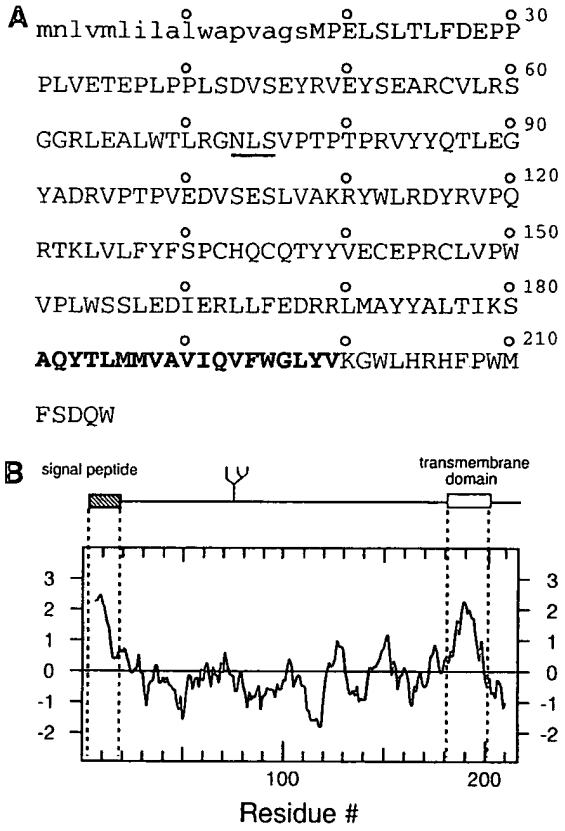
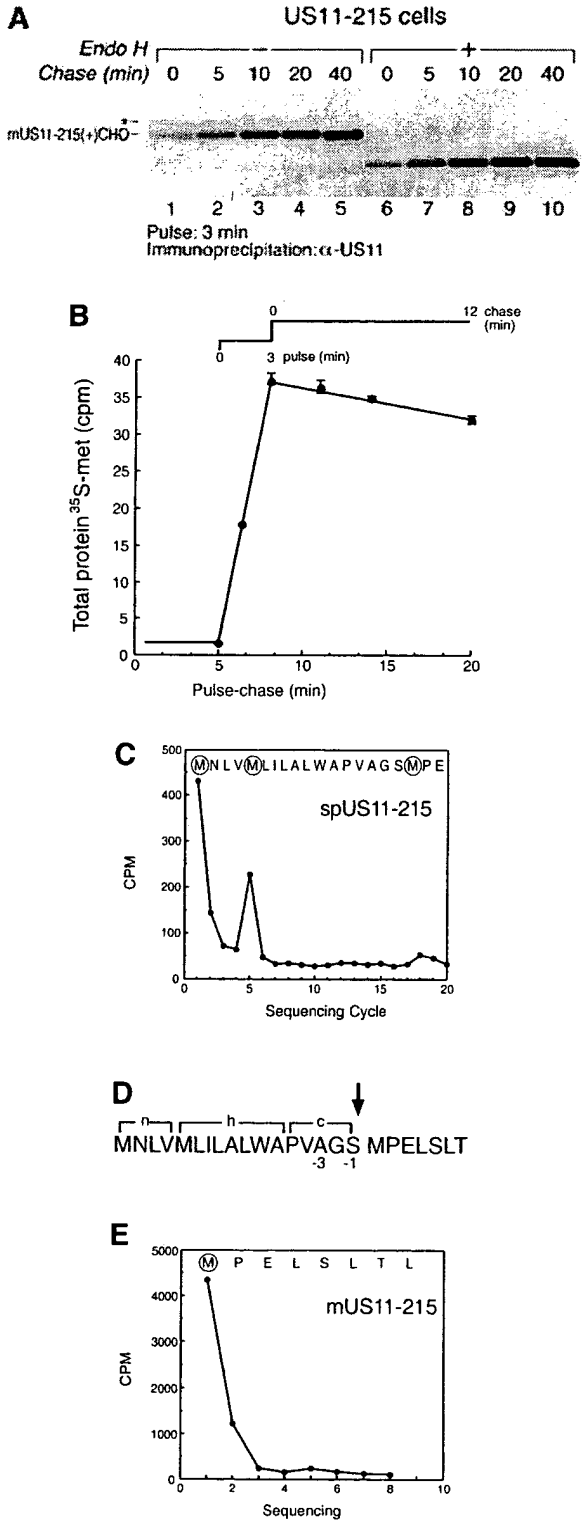


Fig. 1. (**A**) Amino acid sequence (single letter code) of HCMV US11. (**B**) Kyte–Doolittle hydropathy plot of US11. The predicted signal sequence is depicted in lower case. Bold face type represents the predicted transmembrane domain. The N-linked glycosylation site is underlined.

gene product accomplishes this is unclear, but is closely coupled to the biosynthesis of the class I and US11 products. We therefore examined whether the biosynthesis of US11 might reveal unique properties of the ER environment in which US11 normally functions. The maturation of US11 was examined in U373-MG cells stably transfected with US11 (US11-215 cells). US11-215 cells were metabolically labeled for 3 min with [^{35}S]methionine and chased for up to 40 min. The US11 protein was recovered from cell lysates by immunoprecipitation using a polyclonal anti-US11 serum (α-US11) and analyzed by SDS–PAGE (Figure 2A). Two species of US11 of distinct mobility were recovered at early time points (Figure 2B, lanes 1 and 2). The faster moving, major species is the ER-resident, mature form of US11 (mUS11-215). It has a mobility indistinguishable from that of US11 recovered from a microsome-supplemented cell-free translation system (D.Tortorella and H.L.Ploegh, unpublished data).

A precursor–product relationship between the two species was suggested by increased recovery at later chase points of mUS11-215 and decreased recovery of the slower moving species (*) (Figure 2A, lanes 1–4). The identity of the slower moving species (*) was unclear. Is it a distinct form of US11 or is it a protein associated with US11? Both mUS11-215 and the slower moving polypeptide (*) were recovered from SDS-denatured primary

immunoprecipitates in a second round of immunoprecipitation using α-US11 serum (D.Tortorella and H.L.Ploegh, unpublished data). We therefore conclude that the slowly migrating polypeptide is a distinct form of the US11 protein.



The precursor–product conversion observed for the slower moving polypeptide (*) and mUS11-215 does not account fully for the amount of US11 recovered at early chase times. At the early time points of chase, there is a shortfall in the recovery of US11 (Figure 2A, lanes 1–3). This shortfall is not due to the continued incorporation of label during the chase (Figure 2B) and hence must result from the inability to retrieve all US11 at the early time points. Solubilization with the detergent SDS significantly improved recovery of both US11 polypeptides (* and mUS11-215) at the early time points (D.Tortorella and H.L.Ploegh, unpublished data).

Earlier experiments failed to show the presence of endoglycosidase H (Endo H)-resistant US11 and indicated that US11 was confined to the ER, as confirmed by immunoelectron microscopy (Wiertz *et al.*, 1996a). The primary structure of US11 predicts a single N-linked glycan (CHO) attachment site at position 73 (Asn73–Leu–Ser) (Figure 1). Both polypeptides (* and mUS11-215) recovered from the US11 immunoprecipitates were susceptible to digestion by Endo H (Figure 2A, lanes 6–10). The difference between these two molecules of US11 cannot be due to an unusual modification of the N-linked glycan and, therefore, must be caused by differences in the polypeptide backbone.

What type of modification could account for the presence of the slower moving species of US11? Based on the observed apparent molecular weight, the slowly migrating species of US11 may still contain the N-terminal signal peptide (spUS11-215). The polypeptide was isolated from [^{35}S]methionine-labeled cells and subjected to 20 cycles of Edman degradation (Figure 2C). The observed peaks of radioactivity fit the position of the methionines at the N-terminal end of the US11 precursor sequence. These results establish that, surprisingly, the slower moving form (*) (Figure 2A) is indeed a glycosylated US11 molecule that has retained its signal peptide.

The US11 signal peptide contains a typical cleavage site

The factor known to influence signal peptide cleavage is the presence of small amino acid side chains at the –1 and –3 position relative to the cleavage site. Does the US11 signal peptide cleavage site indeed contain the consensus

Fig. 2. Two forms of US11 exist early in biosynthesis. (**A**) US11-215 cells were pulsed for 3 min and chased for up to 40 min. Cells were lysed in 0.5% NP-40 and immunoprecipitated with anti-US11 serum (α-US11). The precipitates were analyzed by SDS–PAGE (12.5%). Two forms of US11 [* and mature US11-215 (+)CHO] were recovered from the US11-215 cell lysates (lanes 1–5). Half of the α-US11 precipitates were digested with Endo H (lanes 6–10). (**B**) Incorporation of [^{35}S]methionine was examined during a pulse–chase experiment of US11-215 cells. TCA-precipitable radioactivity (c.p.m.) from [^{35}S]methionine of each time point was plotted against the pulse–chase experiment. An average of three samples is represented at each value. (**C**) The slower moving US11 polypeptide (*) was subjected to N-terminal radiosequencing. The radioactivity (c.p.m.) from [^{35}S]methionine of each fraction of the N-terminal radiosequencing run was plotted against Edman cycle number. (**D**) The n-, h- and c-regions of the US11 signal peptide are shown. The site of signal peptide cleavage is indicated by an arrow. (**E**) N-terminal radiosequencing of the mature form of US11 (mUS11-215) plotted as radioactivity (c.p.m.) from [^{35}S]methionine versus Edman cycle number.

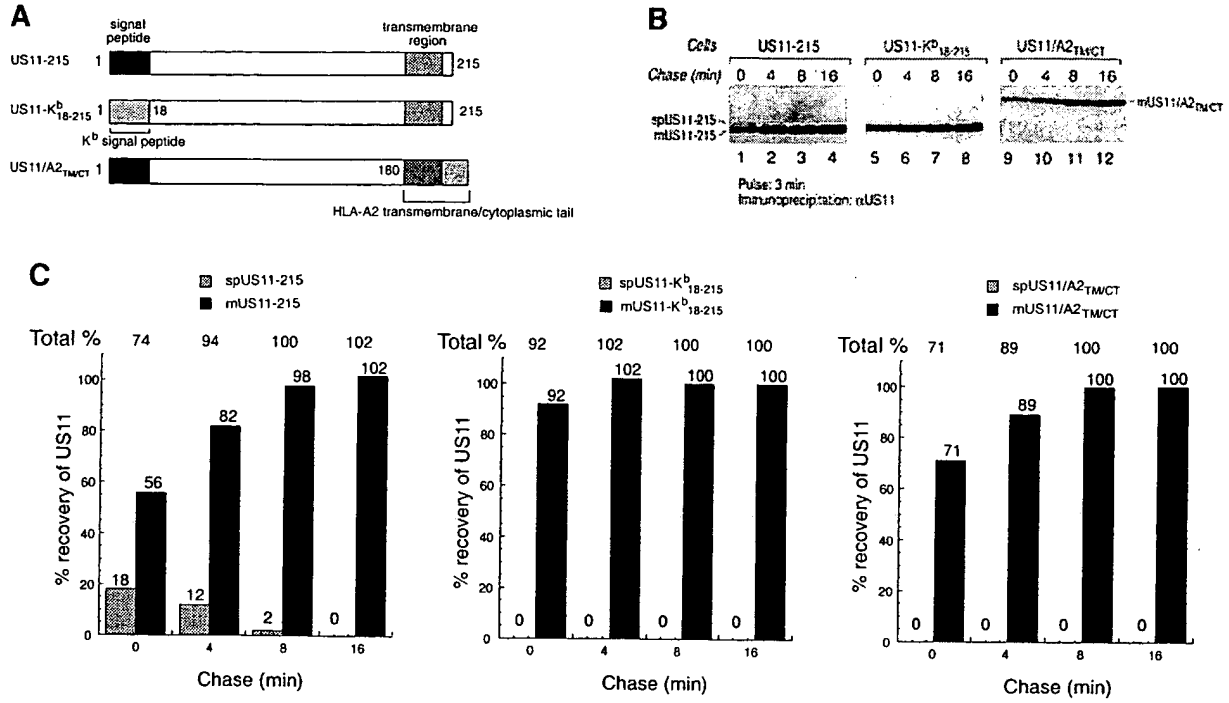


Fig. 3. The delayed cleavage of the US11 signal peptide is determined by its signal sequence and transmembrane/cytoplasmic tail region. (**A**) The US11 chimeric molecules US11–K$^b_{18-215}$, US11/A2$_{TM/CT}$ and wild-type US11-215. (**B**) Processing of these molecules was examined in stable transfectants of MG-U373 cells using pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). The signal peptide-containing form of US11 (spUS11-215) and the mature processed form of US11 (mUS11-215) are indicated. (**C**) The US11-215 molecules recovered from (B) were quantitated by a Molecular Dynamics Storm PhosphorImager. The US11 recovered at each time point is represented as percentage recovery of US11. The US11 recovered at the 8 min chase point was used as the 100% recovery value.

amino acids at the proper position? Analysis of the US11 primary sequence using the SignalP program (www.cbs.dtu.dk/services/SignalP/index.html) (Nielsen *et al.*, 1997a,b) predicts signal peptide cleavage of US11 to occur between residues 17 and 18 (Figure 2D). Serine (17) occurs at position –1 and alanine (15) at position –3, residues that are in perfect agreement with the consensus sequence for a signal peptide cleavage site. Methionine would be the N-terminus of the processed US11 molecule. Indeed, US11 isolated from [^{35}S]methionine-labeled US11-215 cells and subjected to eight cycles of N-terminal sequencing (Edman degradation) yielded methionine at position 1 (Figure 2E). Methionines within the N-terminal sequence of US11 occur at positions 5 and 18. Removal of only four residues from the N-terminus would not account for the mobility difference between the two forms of US11. Therefore, the methionine at position 18 must be the first residue of the mature US11 molecule. These results suggest that the unusual cleavage pattern of the US11 signal peptide is not due to an anomalous signal peptidase cleavage site.

The US11 signal peptide and the transmembrane region contribute to the delayed cleavage of the US11 signal sequence

N-terminal signal peptide cleavage is presumably determined solely by the sequence of the signal peptide itself (Martoglio and Dobberstein, 1998). Changes within the n-, h- or c-region of the signal peptide and the regions directly downstream from the signal peptide affect signal peptide processing (Russel and Model, 1981; Folz and Gordon, 1986; Wiren *et al.*, 1988; Izard and Kendall, 1994). Can the US11 signal peptide itself or regions further downstream of the US11 signal sequence, such as the US11 transmembrane region, play a role in signal peptide cleavage? We generated US11–K$^b_{18-215}$ (Figure 3A), a chimeric molecule in which the US11 signal peptide was replaced with the signal peptide of the murine MHC class I heavy chain H-2K^b, a type I membrane protein. We also generated US11/A2$_{TM/CT}$ (Figure 3A), a chimeric molecule in which the transmembrane and cytoplasmic tail of US11 were replaced with the corresponding regions of human MHC class I heavy chain A2. Cleavage of the H-2K^b signal peptide should now generate the N-terminus of mature US11. Pulse–chase analysis of US11-215 cells shows the recovery of spUS11-215 and mUS11 at the early times points and a precursor–product relationship between the two polypeptides (Figure 3B, lanes 1–4, and C). For neither US11–K$^b_{18-215}$ nor US11/A2$_{TM/CT}$ did we observe the presence of a signal sequence-containing precursor (Figure 3B, lanes 5–8 and 9–12). This result suggests that unique features of US11's signal sequence and transmembrane domain contribute to the persistence of spUS11-215.

The recovery of mUS11-215 and US11/A2$_{TM/CT}$ increases with time (Figure 3B, lanes 1–4 and 9–12, and C). In contrast, recovery of US11–K$^b_{18-215}$ does not significantly change during the chase (Figure 3B, lanes 5–8, and C). We therefore conclude that the US11 signal

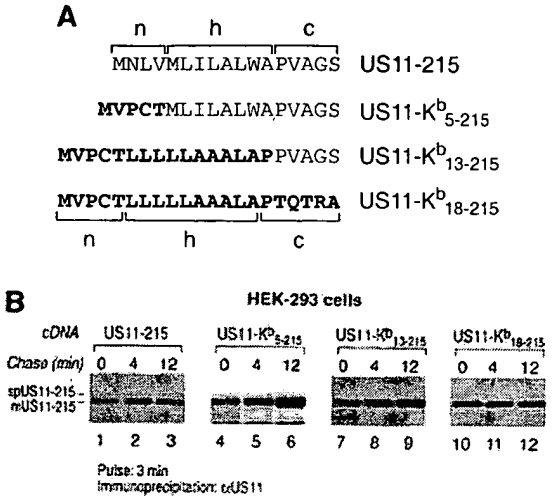


Fig. 4. The n-region of the US11-215 signal peptide is responsible for its delayed cleavage. (**A**) The amino acid sequences of the n-, h- and c-regions of US11-215, US11–K^b_{5-215}, US11–K^b_{13-215} and US11–K^b_{18-215}. Bold letters represent the H-2K^b signal peptide. (**B**) US11–K^b_{5-215} (lanes 4–6), US11–K^b_{13-215} (lanes 7–9) and US11–K^b_{18-215} (lanes 10–12) were transfected in HEK-293 cells and analyzed by pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). The signal peptide-containing form of US11 (spUS11-215) and the mature processed form of US11 (mUS11-215) are indicated.

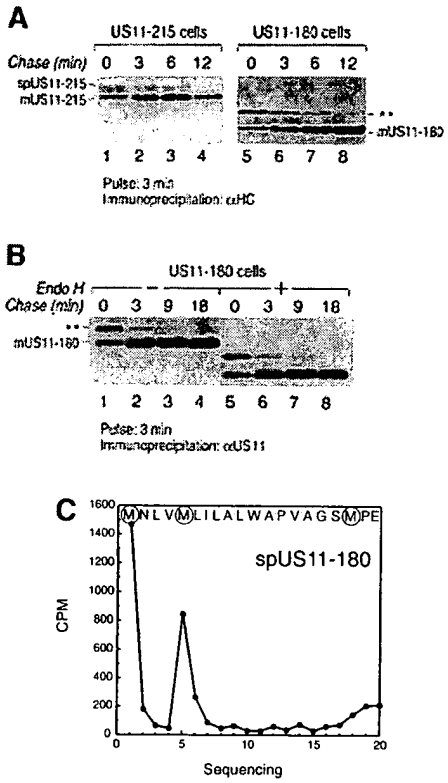


Fig. 5. Signal peptide cleavage of US11-180 is significantly delayed. (**A**) Processing of US11-215 and US11-180 was examined in stable transfectants of MG-U373 cells using pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). The signal peptide-containing form of US11 (spUS11-215) and the mature processed form of US11 (mUS11-215) were immunoprecipitated from US11-215 cells (lanes 1–4). Two major species, ** and the mature processed form of US11-180 (mUS11-180), were recovered from US11-180 cells. (**B**) Half of the α-US11 precipitate recovered from a pulse–chase experiment of US11-180 cells was digested with Endo H (lanes 5–8). (**C**) The slower moving US11-180 polypeptide (**) was subjected to N-terminal radiosequencing. The radioactivity (c.p.m.) recovered at each Edman cycle is shown.

peptide is also responsible for the increased recovery of US11-215 and US11/A2$_{TM/CT}$ at the later time points. We suggest that the manner in which the US11 signal peptide initiates contact with the ER may contribute to its solubility properties.

The n-, h- and c-regions of the US11 signal peptide follow the proposed consensus for a cleavable N-terminal signal peptide. However, the results obtained for the chimeric US11–K^b_{18-215} molecule suggest that the signal peptide itself may account for its delayed cleavage. To characterize further the segment of the US11 signal peptide that is responsible for delayed cleavage, we generated additional chimeras in which the n-region (US11–K^b_{5-215}) or n + h-regions (US11–K^b_{13-215}) of US11 are replaced with the corresponding regions of H-2K^b (Figure 4A). We transfected US11-215, US11–K^b_{5-215}, US11–K^b_{13-215} and US11–K^b_{18-215} into HEK-293 cells and examined their processing by pulse–chase analysis (Figure 4B). For US11-215, a signal peptide-containing form of US11 and the mature form of US11-215 were evident at early chase times (Figure 4B, lanes 1–3). The two polypeptides showed a precursor–product relationship. For the chimeras US11–K^b_{5-215}, US11–K^b_{13-215} and US11–K^b_{18-215}, removal of the signal peptide is rapid and only the mature, cleaved form of US11 is recovered (Figure 4B, lanes 4–12). We conclude that features within the n-region of the US11 signal peptide contribute to its persistence.

During the chase, there is an increase in recovery of the mature form of US11–K^b_{5-215} and US11–K^b_{13-215} (Figure 4B, lanes 4–9), but not for US11–K^b_{18-215} (Figures 4B, lanes 10–12, and 3B, lanes 5–8, and C). Therefore, the c-region of the US11 signal peptide somehow contributes to recovery of mature US11. While the identity of the c-region does not affect the cleavage of the signal peptide, it does contribute to the recovery of mature US11. Perhaps the c-region is responsible for positioning nascent US11 relative to other components of the translocation machinery. This positioning may affect interactions of US11 with other ER components shortly after its completion, and hence its solubility. In contrast, the presence of the full K^b signal sequence neither delays signal peptide cleavage nor affects the recovery of US11 from cell lysates.

The US11 transmembrane region plays a role in US11 signal peptide cleavage

We next examined the role of the US11 transmembrane region in signal peptide cleavage. Such a role was suggested by the analysis of the US11/A2$_{TM/CT}$ chimeric construct (Figure 3). We generated a C-terminal truncation of US11 that lacks the predicted transmembrane segment and the cytoplasmic tail (US11-180) (Figure 1). The

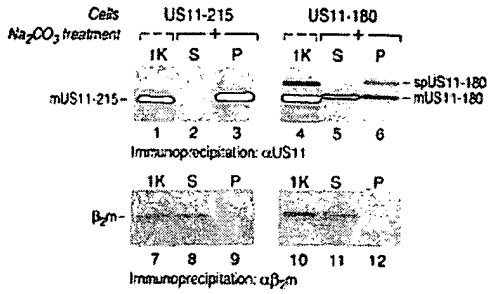


Fig. 6. US11-180 is a soluble molecule. US11-215 and US11-180 cells were metabolically labeled for 15 min. The cells were homogenized with glass beads and centrifuged at 1000 *g*. The 1000 *g* supernatant fractions were treated with 100 mM Na_2CO_3, followed by centrifugation at 150 000 *g*. US11 molecules (lanes 1–6) and β_2m (lanes 7–12) were recovered from the 1000 *g* (1K) pellet, 150 000 *g* supernatant (S) and the 150 000 *g* pellet (P) using α-US11 and α-β_2m serum. The immunoprecipitates were analyzed by SDS–PAGE (12.5%).

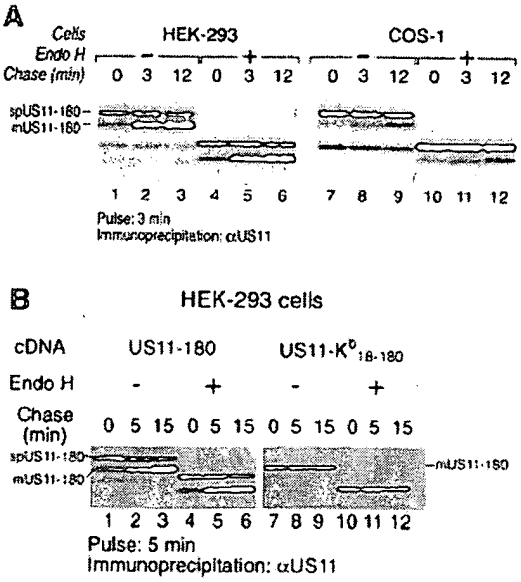


Fig. 7. The US11 signal peptide plays a major role in processing of US11-180. US11-180 cDNA was transfected into HEK-293 and COS-1 cells. (**A**) Processing of US11-180 was examined by pulse–chase analysis. US11-180 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). Half of the immunoprecipitates recovered from the respective transfectants were treated with Endo H (lanes 4–6 and 10–12). (**B**) The US11 signal peptide chimeric molecule US11–K$^b_{18-180}$ and US11-180 were transfected in HEK-293 cells and analyzed by pulse–chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS–PAGE (12.5%). Half of the immunoprecipitates recovered from the respective transfectants were treated with Endo H (lanes 4–6 and 10–12). The signal peptide-containing form of US11 (spUS11-180) and the mature processed form of US11 (mUS11-180) are indicated.

processing of wild-type US11-215 and US11-180 was examined in the appropriate U373-MG transfectants (Figure 5A). US11 recovered at the early chase times from US11-215 cells produced the usual pattern with respect to the precursor–product relationship of spUS11-215 and mUS11-215 (Figure 5A, lanes 1–4). Two major species were recovered from US11-180 cells (** and mUS11-180) (Figure 5A, lanes 5–8). A precursor–product relationship exists for the slower (**) and faster migrating species (mUS11-180) of US11-180. The two polypeptides recovered from the US11-180 transfectants represent distinct forms of the polypeptide backbone and both species of US11-180 are sensitive to Endo H (Figure 5B, compare lanes 1–4 and 5–8).

The slower moving species (**) was isolated from a US11-180 HEK-293 transfectant labeled with [^{35}S]methionine and subjected to 20 cycles of Edman degradation (Figure 5C). The data showed persistence of the signal sequence. The absence of the transmembrane region of US11 thus strongly delays cleavage of its N-terminal signal peptide. An even more pronounced result was observed when US11-180 cDNA was transfected into HEK-293 and COS-1 cells (Figure 7).

mUS11-180 is a soluble protein

The Kyte–Doolittle hydropathy plot of US11 (Figure 1) suggests that the transmembrane region is located between residues 180 and 200. However, the hydrophobic nature of residues 180–200 does not ensure that it is in fact a transmembrane anchor. All attempts at proteolytic removal of the proposed cytoplasmic tail were without success. We performed Na_2CO_3 extractions to explore stable membrane insertion of US11-215 and US11-180 (Figure 6). US11-215 and US11-180 cells were labeled with [^{35}S]methionine and broken with glass beads in the absence of detergent. Homogenates were then centrifuged at 1000 *g* to remove large debris, and the supernatant fraction was treated with 100 mM Na_2CO_3, followed by centrifugation at 150 000 *g* to sediment the extracted microsomes. US11-215 and US11-180 molecules were immunoprecipitated from detergent extracts prepared from the 1000 *g* pellet (Figure 6, lanes 1 and 4), the Na_2CO_3-treated 150 000 *g* soluble fraction (Figure 6, lanes 2 and 5) and the 150 000 *g* pellet fraction (Figure 6, lanes 3 and 6). As a soluble, lumenal control protein, we used β_2-microglobulin (β_2m) (Figure 6, lanes 7–12). The US11-215 polypeptide is recovered exclusively from the 150 000 *g* pellet fraction (Figure 6, lane 3), whereas the bulk of β_2m is recovered from the 150 000 *g* soluble fraction (Figure 6, lane 8). These results confirm that US11-215 is a membrane protein. In contrast, the majority of US11-180 lacking its signal peptide (mUS11-180) and β_2m are recovered from the 150 000 *g* soluble fraction (Figure 6, lanes 5 and 11). These results confirm that mUS11-180 and β_2m are soluble, ER lumenal proteins.

A small fraction of mUS11-180 is recovered from the 150 000 *g* pellet fraction (Figure 6, lane 6) and may represent mUS11-180 that continues to associate with the ER membrane shortly after signal peptide cleavage and prior to its release into the ER lumen. Alternatively, a fraction of mUS11-180 may interact with an ER membrane protein in a Na_2CO_3-resistant manner. As might be expected, the signal peptide-containing form of US11-180 (spUS11-180) remains associated with the membrane fraction even after carbonate extraction (Figure 6, lane 6).

The identity of the signal sequence dictates delayed cleavage of the US11-180 molecule

For reasons of consistency with the data shown earlier, the experiments in Figure 5A were all conducted in U373-MG

cells stably transfected with the US11-180 cDNA. The delayed cleavage of the signal peptide of US11 is not an aberration of the recipient cell line used for transfection. In fact, when we used either HEK-293 or COS-1 cells in a transient transfection protocol, the persistence of the signal peptide-containing form of both US11-215 (Figure 4B, lanes 1–3) and US11-180 (Figure 7A) was much more pronounced. The relative amount of signal sequence-containing precursor of US11-180 was increased to the extreme, such that in COS-1 cells it is in fact the predominant form of US11-180 at the end of the chase (Figure 7A, lanes 7–12). Our data show that the anomalous behavior of the US11 signal peptide is intrinsic to the US11 molecule. In transfection experiments exploiting COS-1 cells to express other type I membrane proteins, the persistence of signal peptides was not observed (Huppa and Ploegh, 1997) and to our knowledge has not been reported by others.

We next addressed the contribution of the signal sequence's identity to the delayed cleavage observed for US11-180. We generated a chimeric molecule, US11–K^b_{18-180}, in which the US11-180 signal peptide is replaced with the H-$2K^b$ signal peptide (Figure 3A). We transfected US11-180 and US11–K^b_{18-180} into HEK-293 cells and examined their processing by pulse–chase analysis (Figure 7B). The immunoprecipitates were treated with Endo H to verify glycosylation and ER insertion (Figure 7B, lanes 4–6 and 10–12). For US11-180 carrying the US11 signal peptide, the signal peptide-containing form of spUS11-180 and the mature processed form of US11-180 were observed throughout the chase (Figure 7B, lanes 1–3). In contrast, a single polypeptide with a mobility similar to that of mUS11-180 is recovered from US11–K^b_{18-180} transfectants (Figure 7B, lanes 7–9). Delayed cleavage of the US11-180 signal peptide no longer occurs when the US11 signal peptide is replaced with the H-$2K^b$ signal peptide. Not only the US11 transmembrane segment, but also features of the US11 signal sequence itself play a major role in US11 signal peptide cleavage.

Discussion

We describe here the unusual properties of the signal sequence of HCMV US11, a type I membrane glycoprotein. Elements contained within the signal sequence's N-terminal segment (Met–Asn–Leu–Val) are responsible for delayed cleavage, such that a fully glycosylated, signal peptide-bearing intermediate is readily detected. In addition, the C-terminal membrane anchor also affects the rate of signal peptide cleavage; a US11 variant lacking its transmembrane/cytoplasmic tail segment (US11-180) shows an even greater delay in signal peptide cleavage than is seen for full-length US11. This effect is at its most extreme in COS-1 cells, where the glycosylated, signal peptide-containing US11-180 protein (spUS11-180) is the majority of US11 polypeptide that persists. To account for these findings, we propose an extended interaction of the signal peptide and transmembrane segment with the processing apparatus.

Conformity with the consensus parameters within the n-, h- and c-regions of the signal peptide predicts proper cleavage of an N-terminal signal peptide. The US11 signal peptide sequence fits the consensus parameters within the n-, h- and c-regions, yet fails to be cleaved efficiently from the nascent chain. Chimeric molecules in which regions (n, n + h or n + h + c) of the US11 signal peptide were replaced with the corresponding regions of the murine class I heavy chain H-$2K^b$ signal peptide demonstrate that it is the n-region of the US11 signal sequence that is mostly responsible for the delayed cleavage of the US11 signal peptide (Figure 4). An irregular n-region has been observed to affect signal peptide processing; a surfeit of positive charges within the n-region of the HIV-1 gp-120 signal sequence probably accounts for its inefficient cleavage (Li *et al.*, 1994, 1996). This aberrant form of gp-120 does not exit the ER and, therefore, cannot be incorporated into a nascent virion. We note that the persistence of the uncleaved signal sequence on gp-120 was never directly shown by sequence analysis.

Regions outside the signal peptide can also influence its cleavage. In pre-pro-apolipoprotein A-II and pre-pro-parathyroid hormone, removal of the propeptide that is immediately downstream of the signal peptide influenced ER protein translocation and proper signal peptide processing (Russel and Model, 1981; Folz and Gordon, 1986; Andrews *et al.*, 1988; Wiren *et al.*, 1988). These changes mostly affect the site of cleavage, shifting it a few residues downstream, while their effect on the rate of signal peptide cleavage was not addressed in any detail. In addition, a mutation at the +2 position of the signal peptide cleavage site of phage coat protein also results in inefficient cleavage (Russel and Model, 1981). All of these mutations are localized immediately downstream of the signal peptide. In contradistinction to such signal sequence-proximal alterations, the transmembrane anchor of US11, at a considerable distance (~160 residues) from the US11 signal sequence, strongly influences signal sequence cleavage. The rate of signal peptide cleavage for the US11 molecule lacking its transmembrane/cytoplasmic tail region (US11-180) is significantly delayed when compared with that seen for wild-type US11 (Figure 5). Replacement of the US11 signal sequence for that of H-$2K^b$ results in rapid processing of US11 lacking the transmembrane segment, such that signal sequence-containing forms are no longer detected. The unprocessed US11-180 polypeptide is probably in an orientation unfavorable for signal peptide cleavage, and the presence of the US11 transmembrane anchor is clearly required for efficient signal peptide processing (Figure 8).

How can the US11 transmembrane anchor accelerate removal of the US11 signal peptide? The transmembrane domain may interact with the signal peptide and position the signal peptide to facilitate access to the cleavage site. Alternatively, the transmembrane anchor may interact with the SPC and enhance recognition of the US11 signal peptide for reasons of physical proximity. While the specificity of signal peptide cleavage is appreciated in terms of the minimum sequence requirements, cleavage itself is a highly regulated process, the dynamics of which are not well understood. The non-catalytic subunits of the SPC have been cloned and isolated, yet their function remains to be determined. Our results show that regulation of signal peptide cleavage may involve *cis*-acting elements within the polypeptide that act at considerable distance

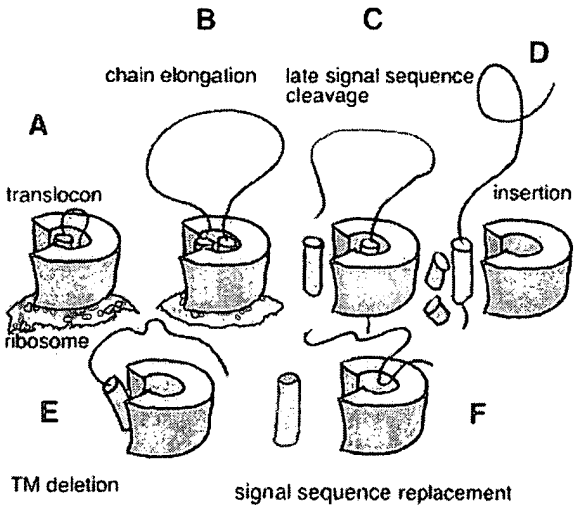


Fig. 8. Model of HCMV US11 signal peptide cleavage. (**A**) The signal peptide (pink) is inserted into the translocon, followed by (**B**) chain elongation of the US11 nascent polypeptide. (**C**) Upon completion of US11 translation, the US11 transmembrane segment (blue) may interact with the signal peptide to delay signal peptide cleavage. (**D**) Upon cleavage of the signal peptide, the US11 molecule inserts into the lipid bilayer; the signal peptide itself may be cleaved further by signal peptide peptidase. (**E**) The signal peptide of a truncated US11 molecule that lacks its transmembrane region and cytoplasmic tail (US11-180) is cleaved inefficiently from the nascent polypeptide. (**F**) Replacement of the US11 signal peptide in US11-180 with the H-2K^b signal peptide (green) results in efficient processing. The US11 transmembrane domain may position the signal peptide in an orientation favorable for cleavage.

from the actual cleavage site. Such elements could perhaps interact with the non-catalytic subunits of signal peptidase.

Immunoelectron microscopy, the maturation status of its single N-linked glycan and the kinetics with which it catalyzes accelerated destruction of class I molecules all place US11 in the ER. The ER environment of the US11 signal peptide may help determine the unusual signal peptide cleavage pattern that we observe. The site of signal peptide cleavage is in the ER and is postulated to be in close proximity to the translocon (Kalies *et al.*, 1998). An intrinsic feature of the US11 signal peptide, more specifically the c-region of the signal peptide, may dictate an association with complexes within the ER as judged from the observed cleavage in detergent extractability (Figure 4 and D.Tortorella and H.L.Ploegh, unpublished data). Shortly after signal peptide cleavage, the recovery of the processed form of US11 increases over the chase period. We suggest that these early biosynthetic forms of US11 may reside in specialized regions of the ER.

To address an issue more peripheral to the central claims of this study: is the cleavage pattern of US11's signal sequence related to US11-induced class I degradation? The signal peptide of the chimeric molecule US11–K$^b_{18-215}$ is cleaved rapidly and this molecule readily supports class I destruction (D.Tortorella and H.L.Ploegh, unpublished data). Therefore, the identity of the US11 signal peptide itself is not essential for the ability of US11 to accelerate class I degradation. The signal peptide of the chimera US11/A2$_{TM/CT}$ is also cleaved rapidly, but class I heavy chains are not degraded in US11/A2$_{TM/CT}$-expressing cells (D.Tortorella and H.L.Ploegh, unpublished data). Deletion of US11's cytoplasmic tail does not abolish degradation of class I heavy chains (D.Tortorella and H.L.Ploegh, unpublished data), and consequently the identity of the transmembrane segment of US11 should be considered essential to its function.

If our interpretation is correct, then perhaps the interaction of the US11 signal peptide and US11 transmembrane segment would help keep the Sec61 complex and its accessories in a configuration that allows recruitment of the class I heavy chains to the translocon. The recorded efficiency of US11-mediated dislocation suggests that the process is tightly linked, temporally and perhaps physically, to protein translocation into the ER. Thus, close proximity of US11 to the translocation apparatus and efficient gating of the protein channel might account for the speed of the dislocation reaction. Ultimately, this aspect must be related to the properties of US11 itself. The unusual maturation of US11, as described here, may turn out be an important aspect of how the dislocation apparatus is put in place.

Materials and methods

Cell lines and antibody

U373-MG astrocytoma cells transfected with the US11-215 cDNA were prepared as described (Jones *et al.*, 1995; Kim *et al.*, 1995) and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 5% calf serum. US11-201, US11-180, US11K$^b_{18-215}$ and US11/A2 cells were maintained in DMEM supplemented with 5% FCS, 5% calf serum and 0.5 mg/ml geneticin (Gibco, Fredrick, MD). The human embryonic kidney cell line (HEK-293) was maintained in DMEM supplemented with 5% FCS and 5% calf serum. The anti-US11 serum was generated by immunizing rabbits with fragments of US11 (amino acids 18–36, 104–122 and 194–210) conjugated to keyhole limpet hemocyanin (Story *et al.*, 1999). The anti-class I heavy chain serum was generated by immunizing rabbits with the bacterially expressed lumenal fragment of HLA-A2 and HLA-B27 heavy chains (Tortorella *et al.*, 1998). The anti-β_2m serum was generated by immunizing rabbits with bacterially expressed human β_2m.

Metabolic labeling of cells and pulse–chase analysis

Cells were detached by trypsin treatment, followed by starvation in methionine/cysteine-free DMEM for 45 min at 37°C. Cells were metabolically labeled with 500 μCi of [^{35}S]methionine/cysteine (1200 Ci/mmol; NEN-Dupont, Boston, MA)/ml at 37°C for the times indicated. In pulse–chase experiments, cells were radiolabeled as above and were chased for the times indicated in DMEM containing non-radiolabeled methionine (2.5 mM) and cysteine (0.5 mM). Cells were then lysed in NP-40 lysis buffer (10 mM Tris pH 7.8, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40) supplemented with 1.5 μg/ml aprotinin, 1 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride (PMSF) followed by immunoprecipitation (see below). For cells lysed in 1% SDS, the SDS concentration was adjusted, prior to immunoprecipitation, to 0.063% with the NP-40 lysis mix.

Immunoprecipitation

Following cell lysis, cell debris was removed by centrifugation at 10 000 *g* for 10 min. Non-specific binding proteins were removed from the cell lysates by the addition of 3 μl/ml normal rabbit serum, 3 μl/ml normal mouse serum and formalin-fixed, heat-killed *Staphylococcus aureus* for 1 h at 4°C. Immunoprecipitation was performed by incubation with antiserum for 45 min at 4°C, followed by the addition of *S.aureus* for 45 min at 4°C. The pelleted *S.aureus* were washed four times with washing buffer (0.5% NP-40 in 50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA). The pellet was resuspended in SDS sample buffer (4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue in 62.5 mM Tris pH 6.8) and the released materials were subjected to 12.5% SDS–PAGE.

cDNA, transfection and Endo H digestion

The cDNA of full-length US11 was cloned from the AD169 HCMV genome using the following primers: 5′ primer, CCGCTCCGAGCGGCGTCGACACCACCATGGAACCTTGTAATGCTTATTCTAGC; 3′ primer, GCTCTAGAGCTCACCACTGGTCCGAAAACATCCAG. The US11 cDNA was cloned into the eukaryotic expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA) using the *Xho–Xba* restriction site in its polylinker region. US11-180 was subcloned from US11 (pcDNA3.1). The chimeric molecules: US11/$A2_{TM/CT}$ [US11(amino acids 1–178)/HLA-A2(amino acids 307–365)]; US11–K^b_{5-215} [H-2K^b(amino acids 1–5)/US11(amino acids 5–215)]; US11–K^b_{13-215} [H-2K^b(amino acids 1–16)/US11(amino acids 13–215)]; US11–K^b_{18-215} [H-2K^b(amino acids 1–21)/US11(amino acids 18–215)]; and US11–K^b_{18-180} [H-2K^b(amino acids 1–21)/US11(amino acids 18–180)] were generated by initially cloning the desired fragment followed by ligation of two of the respective fragments. Using primers specific to the ends of the ligated molecule, it was recloned and inserted into pcDNA3.1. A liposome-mediated transfection (Lipofectamine, Gibco, Fredrick, MD) protocol was performed as described by the manufacturer (4 μg of DNA/20 μl of lipofectamine/10 cm dish of cells). Endo H (New England Biolabs) digestion was performed as described by the manufacturer.

Gel electrophoresis

SDS–PAGE and fluorography were performed as described (Ploegh, 1995). For N-terminal sequencing, the immunoprecipitated US11 protein was resolved by SDS–PAGE and transferred to a PVDF membrane (0.22 μm pore size) in transfer buffer (48 mM Tris-base, 39 mM glycine, 0.037% SDS, 20% methanol) using a semi-dry blotting apparatus (Buchler Instruments, Kansas, MO).

N-terminal sequence analysis

The PVDF membrane that contained the polypeptide of interest was subjected to automated Edman degradation using an Applied Biosystem Protein Sequencer, Model 477, using ATZ chemistry, at the Biopolymers Laboratory at MIT, Center for Cancer Research. The fractions from each degradation sequencing cycle were collected and counted by liquid scintillation spectrometry.

Na_2CO_3 treatment

US11-215 and US11-180 cells were metabolically labeled for 15 min and then washed twice in 50 mM Tris pH 7.5, 250 mM sucrose (homogenization buffer). The cells were resuspended in homogenization buffer and broken by vortexing in the presence of 106 μm glass beads. The homogenate was centrifuged at 1000 *g* for 5 min; the pellet fraction was resuspended in NP-40 lysis mix (see above) and the supernatant was treated with Na_2CO_3 (100 mM final) for 30 min at 4°C (Fujiki *et al.*, 1982). The Na_2CO_3-treated samples were centrifuged at 150 000 *g* using a TLA 100.2 rotor in a Beckman centrifuge. The 150 000 *g* high pH supernatant was adjusted to pH 7 with 1 M HCl and diluted to a final 1× NP-40 lysis mix. The 150 000 *g* pellet was washed twice with homogenization buffer and then resuspended in 1× NP-40 lysis mix. US11 and β_2m were immunoprecipitated from the 1000 *g* pellet, 150 000 *g* supernatant and the 150 000 *g* pellet with the respective antibody.

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